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Uncoupling proteins: current status and therapeutic prospects

Meeting on Uncoupling Proteins

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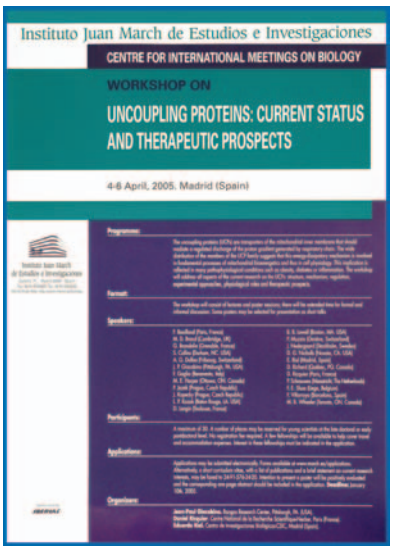

The Meeting on Uncoupling Proteins took place at the Instituto Juan March de Estudios e Investigaciones in Madrid, Spain, between 4 and 6 April 2005, and was organized by E. Rial, D. Ricquier and J.-P. Giacobino.

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Introduction

In a springtime Madrid, under the auspices of the Juan March Foundation, some 50 scientists who work on uncoupling proteins convened to debate the mode of action of these mitochondrial membrane carriers, the control of their expression, their physiological roles and their therapeutic potential.

In bioenergetics, 'uncoupling' refers to any process through which energy released from the combustion of substrate (food) in the mitochondria is not conserved. The final steps in the oxidation of substrate are the transfer of electrons to oxygen, forming water, by the respiratory chain. The energy released is used by the respiratory chain to pump protons out of the mitochondria, as seen in Fig 1A. In most mitochondria, the majority of these protons re-enter through the ATP synthase, and the energy is used to synthesize ATP. However, if the protons re-enter by any other means, the mitochondria are considered to be uncoupled. To some degree this happens in all mitochondria, in ways not understood at present. There is also at least one protein whose function is to allow protons to re-enter the mitochondria without using the energy for any purpose (Fig 1A). Under these conditions, the energy is released as heat. The undisputed uncoupling protein, UCP1, performs this task in brown adipose tissue. Mammals, including newborn humans, use the released heat to protect themselves against cold; this process is referred to as nonshivering thermogenesis. As energy in this process is transferred to heat and not stored as fat in the body, the activity of the uncoupling protein(s) can be viewed as an anti-obesity mechanism—a possibility that has attracted much attention, as both pharmaceutical companies and the general public are looking for easy 'slimming' agents.

It would be expected that the congregation of scientists assembled in Madrid would be united at least in the definition of an uncoupling protein. However, given the intense and animated discussions that characterized the meeting, it is clear that an accord has not even been reached on this basic subject, a fact that underlines the timeliness of this lively meeting.

The reasons for this plurality of definitions are mostly historical. They reflect the development of the field, from a time when only one uncoupling protein was known (UCP1), to the present day when a steadily increasing number of proteins are being advocated as functional uncoupling proteins. The discussions in this respect centred on whether uncoupling proteins should be identified on the basis of their structural similarity to UCP1 or on their functional properties. The advantage of a structural similarity definition is that there is a formal solution to the problem—only proteins with high sequence similarity are included in the UCP family. The disadvantage is that some

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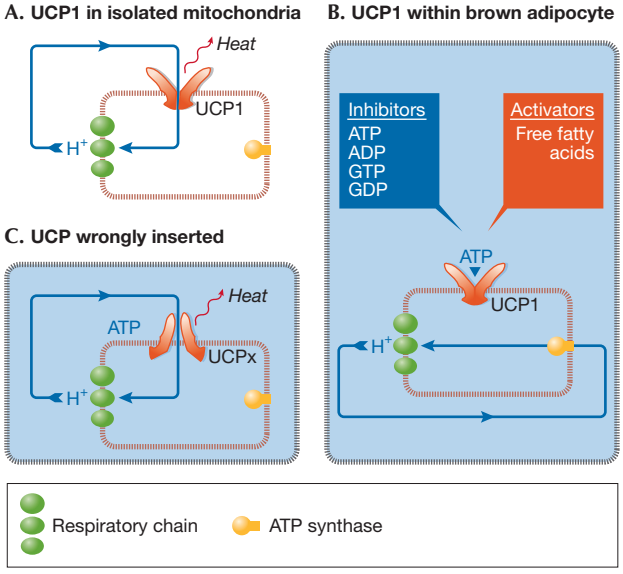

Fig 1 | The function of the original uncoupling protein, UCP1. (**A**) In isolated brown-adipose mitochondria, UCP1 is spontaneously active. Protons pumped out by the respiratory chain leak back through UCP1 and heat is generated. (**B**) Within the unstimulated brown adipocyte, UCP1 is inherently inactive because of constant inhibition by purine nucleotides, particularly ATP. Protons from the respiratory chain can now re-enter the mitochondria, through the ATP synthase—although the activity of this is low in brown adipose tissue. The inhibition of UCP1 can be overcome by fatty acids, which, physiologically, are released from triglycerides in the cell when it is stimulated with norepinephrine. (**C**) If UCP1, or any other UCP, is wrongly inserted into the mitochondrion in transfected cells, it may convey a constantly uncoupled state that cannot be inhibited by ATP.

members of the 'uncoupling protein family' might not be functional uncoupling proteins at all and so uncoupling may be a novelty, evolutionarily introduced by the protein that gave the family its name. Alternatively, the functional definition might reveal uncoupling proteins scattered all over the mitochondrial carrier protein superfamily, making uncoupling a property developed several times during evolution.

Mechanism of action of uncoupling proteins

On the basis of the history of the field, it is natural for us to first summarize the discussions about the mechanism of action of the archetypal uncoupling protein, UCP1, and then to expand from this and consider its nearest structural relatives, UCP2 and UCP3. More distant relatives were only sporadically discussed at the meeting.

Uncoupling protein 1. UCP1 is undoubtedly an uncoupling protein and the only one that has an unchallenged thermogenic function (reviewed in more detail in Cannon & Nedergaard, 2004). Although UCP1 is mechanistically the most studied UCP family member, a consensus concerning its mode of action and the control of its functional activity has not yet been reached. Two basic features have, however, been agreed: purine nucleotides, experimentally often GDP but physiologically ATP and ADP in the cytosol, inhibit UCP1 activity; and its activity is the equivalent of a proton transporter (as is usually shown, see Fig 1). However, whether it is protons that are transported is controversial. UCP1 might instead transport hydroxyl ions (OH^-) or fatty acids. Whether UCP1 needs an 'activator' is also a debated issue—however, it is agreed that an activator is necessary in the cell, with most scientists suggesting that fatty acids are good candidates.

On the basis of its amino-acid sequence, UCP1 can be modelled into the tri-symmetrical structure described elegantly for the closely related adenine nucleotide carrier (G. Brandolin, Grenoble, France)—but this does not immediately reveal a mechanism for its uncoupling function. The crystals of the adenine nucleotide carrier did not contain dimers, which was unexpected as both this carrier and UCP1 had been envisaged as being active in a dimeric form.

The questions discussed at the meeting about the regulation of UCP1 activity reflect the fact that the behaviour of UCP1 depends on the conditions under which it is studied. When UCP1 is present in its cognate environment—that is, in the inner membranes of the mitochondria in a brown-fat cell—it displays no inherent observable uncoupling. Uncoupling (measured as thermogenesis) is only observed when the cells are adequately stimulated, for example, by norepinephrine (Fig 1B). Thus, the mere presence of UCP1 in a cell, expressed entopically or ectopically, does not necessarily lead to a thermogenic effect. Reports that UCP1, without obvious stimulation, causes uncoupling when expressed ectopically, either in white adipose muscle tissue (J. Kopecky, Prague, Czech Republic) or in the heart (F. Bouillaud, Paris, France), could be questioned due to the fact that UCP1 might not be under adequate control in these tissues. However, at least in the heart, ectopic UCP1 is virtually inactive but can be activated by an increase in free fatty acids, for example during reperfusion after transient ischaemia. Endogenously released fatty acids could also activate UCP1 expressed ectopically in white adipose tissue. Similarly, there is no reason to assume that UCP1 in the muscle of a transgenic mouse cannot be activated in a quasi-physiological manner by tissue fatty acids in much the same way as it is activated in brown adipocytes.

By contrast, when mitochondria are isolated from brown adipose tissue, UCP1 activity spontaneously reveals itself (Fig 1A). Isolated brown-adipose mitochondria are inherently uncoupled under standard experimental conditions; that is, they combust substrate, use oxygen and produce heat but no ATP. The models most often discussed in the literature involve the direct participation of fatty acids in the proton-transporting mechanism. However, it was the opinion of several participants at the meeting (in particular, E. Rial, Madrid, Spain, and J. Nedergaard, Stockholm, Sweden) that fatty acids do not participate in the uncoupling process. Instead, the fatty acids function only as anti-inhibitors by relieving the inhibition caused by the purine nucleotides (ATP and ADP) present in the cells—and experimentally by GDP in isolated brown-adipose mitochondria studies (Fig 1B)—prinicpally in accordance with suggestions by Nicholls from the 1970s.

However, in all reconstituted systems examined, UCP1 needs additional fatty acids for activation (P. Jezek, Prague, Czech Republic). Nucleotides inhibit UCP1 activity, but in these systems there is no fatty-acid/nucleotide competition. The relationship between the behaviour of the protein in its natural environment and under reconstituted conditions was not clarified at the meeting.

Uncoupling proteins 2 and 3. The two most UCP1-like proteins, UCP2 and UCP3 (and the closely related avian UCP), were originally identified on the basis of their sequence similarity to UCP1 (for

further reviews on these proteins, see Nedergaard & Cannon, 2003; Rousset *et al*, 2004; Krauss *et al*, 2005). The proposed function of UCP2 and UCP3, as well as the proposed control of their activity, was originally modelled on what was already known for UCP1. In retrospect, we may have been naive in expecting that these proteins, with only 58% amino-acid sequence similarity to UCP1, should have an identical biochemical function to that of UCP1. Indeed, evidence for the expected thermogenic effect of UCP2 and UCP3 has been weak, and even the assumption that these proteins function as uncoupling proteins was rightfully challenged at the meeting. One can hypothesize that UCP2 and UCP3 will have functions more related to each other than to UCP1, based not only on their high sequence similarity, but also on the fact that their juxtaposed chromosomal locations indicate that they developed from a common ancestral gene by unequal crossing over.

The point that within any cellular system, a UCP is never 'uncoupling' without adequate activation was stressed by D. Nicholls (Novata, CA, USA) for UCP1 and repeatedly advocated for UCP2 and UCP3 by M. Brand (Cambridge, UK). The logical extrapolation of this view is that any effect of inherent uncoupling that has been shown for ectopically expressed UCPs in the absence of stimulation—for example, prolongation of generation time in yeast or membrane potential decrease in mitochondria within unstimulated cells—is an artefact caused by a significant fraction of the UCP being inserted incorrectly into the membrane (Fig 1C). Through this incorrect insertion, the membrane becomes constantly leaky. Similarly, all observations in which the ectopic expression of UCPs has been reported to have thermogenic and 'slimming' effects probably constitute experimental artefacts. In this respect, the effects of ectopic expression of a UCP are the same as if mice or men were to eat a chemical uncoupler such as dinitrophenol (DNP)—that is, chemically allowing an unregulated re-entry of protons into the mitochondria.

Given that UCP2 and UCP3 are probably not thermogenic, other suggestions for their function have been advocated. An extension of the definitely disputed idea that UCP1 is a fatty-acid carrier (see above), is the possibility that UCP3 extrudes fatty acids trapped in the mitochondrial matrix in a free form, and not in the metabolizable CoA ester form (P. Schrauwen, Maastricht, The Netherlands, and M.-E. Harper, Ottawa, Canada). Related to this possibility is the idea that UCP3 is involved in the control of substrate use—fatty acids versus glucose—without necessarily being a fatty-acid transporter itself (A. Dulloo, Fribourg, Switzerland).

The most discussed hypothesis at the meeting was that UCP2 and UCP3 do indeed function as uncoupling proteins, but only when oxidative stress (superoxide production) can be ameliorated by their activity. This is generally presented as the 'mild-uncoupling' hypothesis (Fig 2). It was debated whether this type of 'not thermogenic but still membrane potential lowering activity' is bioenergetically possible. Connected to this is the issue of very low amounts of UCP2 and UCP3 in mitochondria. There is probably at least 200-fold less UCP3 in muscle mitochondria than UCP1 in brown-adipose mitochondria. As the amount of UCP1 is rate-limiting for uncoupling in brown-adipose mitochondria, it is difficult to understand how such a small amount of UCP2 or UCP3 could lead to observable uncoupling. This could also be why no uncoupling effects were seen even with a fourfold increase in the amount of UCP3 in muscle mitochondria (I. Shabalina, Stockholm, Sweden, and S. Cadenas, Madrid, Spain).

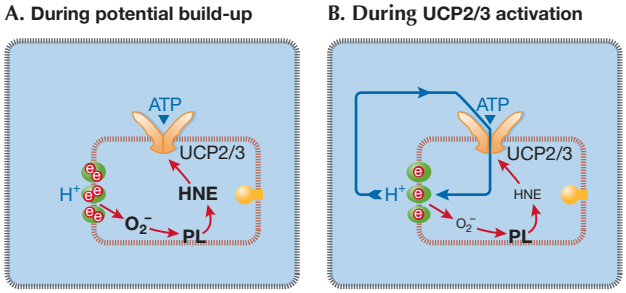

Fig 2 | The suggested function of uncoupling proteins 2 and 3 as protectors against the formation of reactive oxygen species. This figure is based on the hypothesis of M. Brand (Cambridge, UK), which was critically debated during the meeting. (**A**) In conditions under which electrons build up in the respiratory chain, for example when ATP synthesis is not ongoing, electrons might interact with oxygen to form superoxide, leading to oxidative damage. However, superoxide could activate UCPs through the formation of hydroxynonenal (HNE) from phospholipid acyl chains (PLs). (**B**) Activated UCP could dissipate some of the proton motive force, so that fewer electrons accumulate, leading to less superoxide production.

The oxidative-stress protection hypothesis was challenged by the absence of UCP2 and UCP3 in some tissues, such as in liver and certain brain areas, but it has been shown that UCP2 is expressed in these tissues under more stressful conditions. Nicholls pointed out that UCP2 and UCP3 often seem to aggravate stress rather than relieve it. However, the oxidative-stress protection function is supported by the observation that macrophages from UCP2-null mice produce more superoxide, which results in a chronic activation of the NF-κB system with expected inflammatory consequences (S. Collins, Research Triangle Park, NC, USA). In addition, mice without UCP2 are more susceptible than normal mice to chemically induced colon cancer. A total of four tumours were found in ten two-year-old UCP2-ablated mice, but not a single wild-type littermate had developed a tumour by that age (Z. Derdák, Providence, RI, USA). This information should trigger more dedicated investigations into a possible protective function of UCP2, using mice with identical genetic backgrounds living under normal conditions. Clear cause-and-effect associations between UCP-based mechanisms for the control of reactive oxygen species activity and subsequent pathology have yet to be established.

The most mechanistic formulation of the oxidative-stress protection hypothesis was given by Brand, who presented the updated version of the functional scheme that has been progressively developed from studies in his laboratory (Fig 2; Brand *et al*, 2004). Brand suggested that the UCPs—whether or not this includes UCP1 is still open—specifically protect against oxidative damage caused by fatty acids, particularly polyunsaturated fatty acids from membrane phospholipids. These fatty acids can be attacked by mitochondrially-generated superoxide that converts them into 4-hydroxy-2-nonenal (HNE) and then interacts with the UCPs to make them able to conduct protons (or an equivalent). This 'mild uncoupling' would decrease the membrane potential and thus diminish the rate of production of superoxide; that is, this would be a self-regulating protective system. The physiological validity of this theory was discussed, with particular emphasis on the need for a more physiologically

relevant assay—for example, using endogenous sources of superoxide from the cell or the mitochondrion itself to induce uncoupling activity in mitochondria in the presence of millimolar concentrations of ATP. Experiments performed under slightly different conditions have not all confirmed the hypothesis, but Brand pointed out that the chain of processes involved may have had insufficient time to develop in these experiments. A covalently modified UCP should be generated if superoxides activate the UCP, but such a modified UCP has not been detected so far. A controversial but important detail concerns the presence or absence of UCP2 and UCP2-related effects in kidney mitochondria—if UCP2 is not found there, can there then be UCP-related effects? However, this controversy may be due to differences in UCP2 expression according to mouse strain, gender or other conditions. Thus, whether the activation by HNE represents a genuine physiologically relevant process or merely a state that can be biochemically induced under specified conditions is still open to debate.

Control of expression of uncoupling proteins

As the functional studies of the theoretical UCPs, particularly UCP2 and UCP3, were not successful in defining UCPs or their physiological significance, there were expectations that the presentations about the regulation of expression of these proteins could provide convincing clues.

The tissue distributions of the three mammalian UCPs are distinct (D. Ricquier, Paris, France, and Bouillaud). As detailed below, UCP1 and UCP3 are virtually tissue-specific (UCP1 in brown adipose tissue; UCP3 in muscle and brown adipose tissue), whereas UCP2 is expressed in many different tissues. In particular, UCP1 gene expression is restricted to the brown adipocytes that contain a spectacularly large number of mitochondria, whereas UCP2 and UCP3 are present in cells that have relatively few of these organelles. Schrauwen even reported that in skeletal muscle, UCP3 is essentially only expressed in the type IIb glycolytic fibres, which indeed might be the fibres that need protection from fatty acids.

Uncoupling protein 1. In mammals, UCP1 is found only in brown adipose tissue. Unexpectedly, a synteny investigation—that is, identification based on identical chromosome location—has revealed the existence of a protein that might be an evolutionary ancestor having 70% amino-acid sequence similarity to UCP1; this protein is expressed in the livers of fish (M. Jastroch, Marburg, Germany). For UCP1-based thermogenesis to become a method for reducing obesity in humans, as has been envisaged, it will probably be essential to induce its expression in tissues other than brown adipose tissue. Many studies have identified a pivotal role for peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) in the regulation of UCP1 gene transcription. D. Langin (Toulouse, France) showed that the introduction of PGC-1α into cultured human white adipocytes can activate brown adipogenesis in these cells. This is consistent with genetic studies in the mouse that were described by L. Kozak (Baton Rouge, LA, USA), which delineated the complex discussion of the possible conversion of white to brown adipocytes upon adrenergic stimulation *in vivo*. Collins described studies showing that activation of PGC-1α and UCP1 gene transcription by adrenergic stimulation proceeds through a p38 mitogen-activated protein (MAP) kinase pathway that is associated with the JNK-interacting protein (JIP) family of scaffold proteins. On the basis of their work with the β-adrenergic receptor knockout (KO) mouse, J.-P. Giacobino (Pittsburg, PA, USA) and L. Lehr (Geneva, Switzerland) challenged the pivotal role of PGC-1α in the control of UCP1 gene expression, as they reported that these mice have a 93% reduction in UCP1 mRNA despite normal levels of PGC-1α. Addition of the PPARγ agonist rosiglitazone to such cells rescues UCP1 expression, which emphasizes the importance of activated PPARγ in the control of UCP1 expression. In this regard, brown adipocytes recruited in white adipose tissue during exposure to cold differ from genuine brown adipocytes. Rosiglitazone more efficiently induces UCP1 expression in progenitors from white than from brown adipose tissue. A detailed characterization of similar progenitors of brown adipocytes in human white adipose tissue might help to find ways to stimulate thermogenesis and fat oxidation in humans, with possible slimming effects.

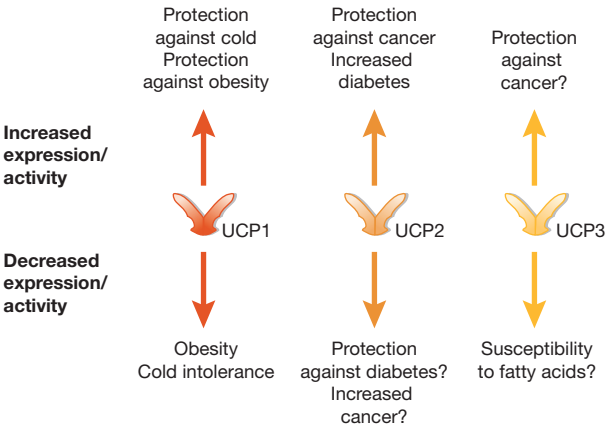

Fig 3 | Pathological consequences and therapeutic effects of altered expression/activity of the different uncoupling proteins.

Uncoupling protein 2. UCP2 mRNA has been detected in macrophages, lymphocytes, thymocytes, pulmonary cells, enterocytes, adipocytes, pancreatic β-cells and certain neurons and, at a lower level, in liver, muscle and kidney cells. In the brain, UCP2 gene expression is generally low but high levels of UCP2 mRNA have been found in some regions, such as the limbic system and particular subdomains of the hypothalamus (D. Richard, Quebec, Canada). In these areas, UCP2 mRNA is present in neurons that produce corticotropin-releasing factor or arginine-vasopressin, as well as in the arcuate nucleus—a brain region involved in the control of food intake. Indeed, Richard observed changes in UCP2 KO mice that suggest a role for UCP2 in the control of food intake. In agreement with a role for UCP2 in neuroprotection, proposed by several teams, Richard also observed that kainic acid, an excitotoxic substance, induces UCP2 expression in the hippocampus.

UCP2 synthesis in macrophages—as well as in adipocytes and pancreatic β-cells—is under the control of PPARγ, liver X receptor (LXR) and sterol regulatory element-binding protein 1 (SREBP-1c; Collins). UCP2 mRNA levels in epididymal fat are markedly increased after fasting. Whether this UCP2 elevation could contribute to the ATP depletion observed in adipose cells during starvation is unknown (Kopecky).

Uncoupling protein 3. UCP3 expression levels in the skeletal muscle of animals or humans respond to changes in fatty-acid flux (F. Villarroya, Barcelona, Spain; Harper; Dulloo; Schrauwen). In mouse pups, the appearance of UCP3 in skeletal muscle just after birth is linked to lipid intake (Villarroya). MyoD, PPARα and PPARδ positively regulate the human UCP3 promoter, and the histone acetylase activity of p300 promotes PPARα-dependent activation (Villarroya). Pharmacological inhibition of fatty-acid oxidation induces an increase in UCP3 levels, whereas the restoration of fat oxidation by riboflavin downregulates UCP3, which indicates that UCP3 is upregulated when long-chain fatty-acid delivery exceeds fatty-acid oxidation capacity (Schrauwen). The thyroid hormone triiodothyronine has a positive role in the control of UCP3 expression (F. Goglia, Benevento, Italy).

Physiological function and therapeutic potential

The physiological function and therapeutic potential of the UCPs are summarized in Fig 3. UCP1 has been shown to be crucial for maintaining body temperature, as UCP1 KO mice are cold sensitive. Nedergaard and Kozak described experiments in which UCP1-deficient mice have been used to examine the presence of alternative thermogenic mechanisms that these mice can use to maintain their body temperature. Such studies are of interest as they could lead to the identification of novel thermogenic mechanisms that are relevant to energy balance in humans. Whereas Nedergaard is convinced that the UCP1-deficient mouse uses only shivering thermogenesis to protect its body temperature, Kozak described results suggesting that mechanisms associated with Ca^{2+}-ion cycling in muscle and adipose tissue are induced in these mice to tolerate the cold. Leptin might also participate in this process, as mice deficient for both leptin and UCP1 cannot adapt to the cold unless they are injected with this hormone. Dulloo showed that leptin stimulates oxygen consumption in isolated muscle fibres by a futile substrate lipid cycling that could be a thermogenic mechanism in the regulation of body weight.

The phenotypes of mice with inactivated *UCP2* or *UCP3* genes are not related to either defective body temperature or body weight regulation. Reduced expression of UCP2 seems to favour enhanced insulin secretion in the pancreas and increased resistance to infection by microbial pathogens. However, the indication that UCP2 protects against tumours implies that a general reduction of UCP2 activity might have undesirable side effects.

The phenotypes resulting from over- or under-expression of UCP3 are vague, and so UCP3 has no therapeutic implications at present.

Conclusions and perspectives

The lively discussions throughout the workshop provide evidence of the passionate opinions among the participants. The physiological functions of UCP2 and UCP3, which are only vaguely discernible at present, seem different from the evident thermogenic role of UCP1. Determining the function of UCP2 and UCP3 and the structural basis for uncoupling by UCP1 will keep discussions going for many years to come—there is clearly an uncoupling protein family, both with respect to the proteins and the scientists involved. Future meetings on uncoupling proteins will undoubtedly reinforce the combination of congeniality and individuality that is typical of family reunions.

ACKNOWLEDGEMENTS

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Jan Nedergaard, Leslie P. Kozak and Daniel Ricquier, photographed on Plaza Mayor in Madrid during a break in the meeting.